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Changing a Single Amino Acid in *Clostridium perfringens* β -Toxin Affects the Efficiency of Heterologous Secretion by *Bacillus subtilis*[▽]

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Achieving efficient heterologous protein production and secretion by *Bacillus subtilis* is an attractive prospect, although often disappointingly low yields are reached. The expression of detoxified *Clostridium perfringens* β -toxin (β -toxoid) is exemplary for this. Although β -toxin can be efficiently expressed and secreted by *Bacillus subtilis*, the genetically detoxified, and industrially interesting, β -toxoid variant is difficult to obtain in high amounts. To optimize the expression of this putative vaccine component, we studied the differences in the global gene regulation responses of *B. subtilis* to overproduction of either β -toxin or β -toxoid by transcriptomics. A clear difference was the upregulation of the CsxRS regulon, known to be induced upon secretion stress, when β -toxoid is produced. YkoJ, a protein of unknown function, was also upregulated, and we show that its expression is dependent on csxS. We then focused on the heterologous protein itself and found that the major secretion bottleneck can be traced back to a single amino acid substitution between the β -toxin and the β -toxoid, which results in the rapid degradation of β -toxoid following secretion across the cytoplasmic membrane. In contrast to β -toxin, β -toxoid protein is more prone to degradation directly after secretion, most likely due to poor folding characteristics introduced with point mutations. Our results show that although the host can be adapted in many ways, the intrinsic properties of a heterologous protein can play a decisive role when optimizing heterologous protein production.

Bacillus subtilis is widely used for protein production and secretion (12). Nevertheless, the secretion of heterologous proteins is often problematic, and many attempts have been made to overcome the poor production of secreted heterologous proteins, with varying success. A major problem encountered when using *Bacillus subtilis* as a production platform is protein degradation by extracellular proteases secreted by this organism. By using extracellular protease-deficient strains, this problem can be reduced to some extent, but such strains are more prone to lysis and have a reduced growth rate (34).

Another bottleneck is the rapid degradation of the secreted protein by a quality control mechanism in the cell wall environment (3, 14, 27, 30). Typically, this degradation takes place before the protein can fold into its native and usually protease-resistant conformation. *B. subtilis* responds to the overexpression of secreted proteins with the so-called secretion-stress response. CsxRS controls this stress response and regulates the expression of HtrA and HtrB, two serine proteases that also can act as chaperones (7). Secretion stress is thought to be triggered by unfolded proteins at the *trans* side of the membrane due to problems that occur in late stages of protein secretion (19), presumably as a consequence of slow folding at the membrane cell wall interface (5, 13).

To promote correct and rapid folding of the secreted heterologous proteins, several measures can be taken. Expression of

chaperones and proteases can be altered (33), the charge of the cell wall or the secreted protein can be adapted (28), and the availability of divalent metal ions can accelerate folding of the proteins (27). However, such measures usually improve secretion by only a factor of 1.5 to 3.

In this work, we report on important factors influencing the secretion of β -toxoid, a genetically inactive form of *Clostridium perfringens* β -toxin. This protein is of industrial interest since it is a major component in vaccine preparations protecting against *C. perfringens* type B and C infections. The wild-type (WT) *C. perfringens* β -toxin is a potent toxin that requires chemical deactivation before it can be used as a safe vaccine component. Point mutations have been introduced that render this toxin no longer toxic but still immunogenic. However, this altered β -toxoid is very poorly secreted by *B. subtilis* (21).

In an attempt to identify the bottleneck in secretion of β -toxoid, we compared levels of global gene expression during the overproduction of β -toxin and β -toxoid. We tested whether altering the expression of the strongest upregulated gene could improve secretion yield. Unfortunately this did not yield the desired results.

Strikingly, the wild-type β -toxin protein can be efficiently secreted by *B. subtilis*. We therefore focused on the protein itself and analyzed the specific effects of the amino acid substitutions that differ between β -toxin and β -toxoid. Surprisingly, this revealed that only a single amino acid residue dictates the difference between high and very poor secretion yields.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Lactococcus lactis* strains were grown at 30°C in M17 broth with 0.5% glucose (GM17) (29). *B. subtilis* strains were

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TABLE 1. Strains and plasmids used in this study^a

Strain or plasmid	Genotype and description	Source or reference
Strains		
<i>B. subtilis</i> 168	<i>trpC2</i>	1
<i>B. subtilis</i> NZ8900	<i>trpC2 amyE::spaRK</i>	4
<i>B. subtilis</i> WB800	<i>nprE aprE epr bpr mpr::ble nprB::bsr Δvpr wprA::hyg</i>	34
<i>B. subtilis</i> NZ8900-Δ <i>ykoJ</i>	<i>trpC2 amyE::spaRK ykoJ::spcR</i>	This study
<i>B. subtilis</i> HT100A	NZ8900 containing <i>PhtrA</i> -GFP fusion integrated in the chromosome	This study
<i>B. subtilis</i> Py <i>koJ</i> -GFP strain	NZ8900 containing Py <i>koJ</i> -GFP fusion integrated in the chromosome	This study
<i>L. lactis</i> MG1363	Plasmid-free strain	10
<i>L. lactis</i> NZ9000	<i>penN::NisRK</i>	16
Plasmids		
pSG1151	<i>bla cat gfp</i>	20
pPy <i>koJ</i> -GFP	pSG1151::Py <i>koJ</i> -gfp	This study
pPh <i>trA</i> -GFP	pSG1151::Ph <i>trA</i> -gfp	This study
pDG1726	Vector containing spectinomycin resistance cassette	11
pXB10	pUB110 containing β-toxin-coding region	25
pNZ8048	Nisin vector, Em ^r	9
pNZbtotoxoid (pNZbttox)	pNZ8048::β-toxoid	21
pNZbtotoxin	pNZ8048::β-toxin	This study
pNZ8901	Cloning vector containing subtilin-inducible promoter, Cm ^r	4
pNZ8903	Cloning vector containing WT subtilin-inducible promoter, Em ^r	4
pNRS-ykoJ	pNZ8901 containing <i>ykoJ</i> gene	This study
pNRS-βtoxooid (AAA)	pNZ8903 containing β-toxooid gene	This study
pNRS-βtoxin (DDK)	pNZ8903 containing β-toxin gene	This study
pNRS-βtox-DAA	pNZ8903 containing β-tox-DAA gene	This study
pNRS-βtox-ADA	pNZ8903 containing β-tox-ADA gene	This study
pNRS-βtox-AAK	pNZ8903 containing β-tox-AAK gene	This study
pNRS-βtox-DDA	pNZ8903 containing β-tox-DDA gene	This study
pNRS-βtox-DAK	pNZ8903 containing β-tox-DAK gene	This study
pNRS-βtox-ADK	pNZ8903 containing β-tox-ADK gene	This study
pRNΔ <i>ykoJ</i> _Sp	pUC18 containing up- and downstream regions of <i>ykoJ</i> flanking a spectinomycin resistance cassette	This study

^a See Materials and Methods and legends to Fig. 1 and 5 for explanation of β-toxin/β-toxooid gene designations.

grown at 37°C under vigorous agitation in TY (1% tryptone, 0.5% yeast extract, 1.0% NaCl) or minimal medium (25). For the selection of transformants, appropriate antibiotics were added to the growth media at the following concentrations: chloramphenicol, 5 μg/ml; spectinomycin, 100 μg/ml; hygromycin, 125 μg/ml; and erythromycin, 5 μg/ml.

Strain constructions and transformation. The cloning and transformation procedures were performed according to established techniques (6, 23) and suppliers' manuals. Restriction enzymes, DNA polymerases, deoxynucleotides, and T4 DNA ligase were obtained from Roche Diagnostics (Mannheim, Germany) and Fermentas Life Sciences (Vilnius, Lithuania) and used as specified by the suppliers. Table 2 lists the nucleotide sequences of primers used for PCR.

Inducible β-toxin and *ykoJ* plasmids. To construct the *B. subtilis* subtilin-inducible plasmids pNRS-βtoxooid and pNRS-βtoxin, the β-toxooid gene was amplified by PCR from the pBtox-1 plasmid, and the β-toxin gene was amplified from plasmid pXB10, respectively, using primers Btox-fw, BstEII and Btoxoid-RV2-XhoI. These PCR products were digested with BstEII and AvaI and ligated into the likewise digested replicative vector pNZ8903 containing the unmodified subtilin-inducible *spaS* promoter (4). To construct pNRS-ykoJ, the *ykoJ* gene was amplified by PCR from *B. subtilis* 168 chromosomal DNA using primers ykoJ-fw1 and ykoJ-rv1, digested with BstEII and AvaI, and ligated into the likewise digested replicative vector pNZ8901 containing the subtilin-inducible *spaS* promoter (4).

To construct the *L. lactis* plasmids pNZ-βtoxooid and pNZ-βtoxin, the β-toxooid gene was amplified by PCR from the pBtox-1 plasmid and the β-toxin gene was amplified by PCR from plasmid pXB10 using primers Btoxoid-RN2-fw and Btoxoid-RN2-rv. This product was digested with NcoI and AvaI and ligated into the likewise digested replicative vector pNZ8048 containing the nisin-inducible promoter (9).

Ligation mixtures were transferred to electrocompetent *L. lactis* MG1363 culture or *L. lactis* NZ9000 culture using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA), as described previously (18). Colonies were selected on solid

medium for the erythromycin resistance. Isolated plasmids were checked for correct ligation by AvaI-AvaII digestion and DNA sequencing.

B. subtilis NZ8900 was transformed with the constructed replicative plasmids isolated from *L. lactis* and selected on solid medium for appropriate resistance.

***ykoJ* deletion construct.** Upstream and downstream regions of the *ykoJ* gene were amplified by PCR using primers up_ykoJ-fw1 and up_ykoJ-rv1 and down_ykoJ-fw1 and down_ykoJ-rv1, respectively, and the resulting PCR products were digested with PstI plus HindIII and HindIII plus XbaI, respectively. These products were ligated into a four-point ligation to both sides of a HindIII-digested spectinomycin resistance cassette, obtained by PCR using pDG1726 as a template and primers RNlacZ-fw and RNlacZ-rv, and a PstI-plus-XbaI-digested pUC18 plasmid. The resulting plasmid, pRNΔ*ykoJ*_Sp, was amplified with *Escherichia coli* and transformed to *B. subtilis* NZ8900 to create NZ8900-Δ*ykoJ*. Colonies were checked for integration of the spectinomycin resistance cassette via double crossover at the locus of *ykoJ* by PCR.

Intermediate β-toxin mutants. All β-toxin variants were constructed by PCR on the template plasmids pNRS-βtoxin (to create pNRS-βtox-A₅₄DK, -A₅₄DA, and -A₅₄AK) and pNRS-βtoxooid (to create pNRS-βtox-D₅₄AK, -D₅₄DA, and -D₅₄AA). The plasmids were amplified using a forward primer annealing next to the mutagenesis target site and containing an Eco31I recognition site and a specific reverse primer containing the desired point mutation and an Eco31I site (Table 2). After amplifying the whole plasmid, the PCR product was digested with Eco31I and circularized by self-ligation. The resulting plasmid was electroporated to *L. lactis* MG1363. Mutants were checked for the appearance/disappearance of the ClaI site contained within the first codon of the DDK region and subsequently checked by DNA sequencing (Baseclear, Leiden, The Netherlands). Correctly constructed plasmids were transformed to *B. subtilis* NZ8900 and selected for erythromycin resistance.

Protein expression, protein isolation, gel electrophoresis, and Western blotting. *B. subtilis* cultures were diluted from an overnight culture to a starting optical density at 600 nm (OD₆₀₀) of 0.1. β-Toxin or β-toxooid expression was

TABLE 2. Primers used in this study

Primer name	Description ^a	Sequence (5' to 3')
Btoxoid-RN2-fw	Forward primer for β -toxoid cloning in pNZ8048	CGTTGCCATGGAGAAAAAATTTATTTTCATTAGTTATAG
Btoxoid-RN2-rv	Reverse primer for β -toxoid cloning in pNZ8048	CGCTCTAGATTAAATAGCTGTTACTTTGTGAG
Btox-fw_BstEII	Forward primer for Btoxoid SS, introduces BstEII and NdeI sites	TCGGTGACCCATATGAAGAAAAAATTTATTTTCATTAG
Btoxoid-RN2-XhoI	Reverse primer for β -toxoid, introduces XhoI site	CGCCTCGAGTTAAATAGCTGTTACTTTGTGAG
RNlacZ-fw	Universal primer on pUC18-derived MCS	GTGAGCGGATAACAATTTACACAGG
RNlacZ-rv	Universal primer on pUC18-derived MCS	GGTTTCCAGTCACGACGTTGTAA
PhtrA_fw-KpnI	Forward primer for promoter region <i>htrA</i> , introduces KpnI site	CGTGAGGTACCGGCTTCTGTTTCTGCC
PhtrA_rv	Reverse primer for promoter region <i>htrA</i> , introduces HindIII site	CATCACAAGCTTATCCATCATGTTCACTCCG
PykoJ-fw1	Forward primer for amplification of <i>ykoJ</i> promoter, introduces KpnI site	CTGGTACCGCAGTGAATCCATCTGCCATGAC
PykoJ-rv1	Reverse primer for amplification of <i>ykoJ</i> promoter, introduces HindIII site	ACTAAGCTTGAGCAATTTGTGAGCCCTCCTTTGT
ykoJ-fw1	Forward primer to amplify <i>ykoJ</i> for overexpression, introduces BstEII site	TGTGGTGACCCAAATGCTCAAGAAAAATGGATGGTC
ykoJ-rv1	Reverse primer to amplify <i>ykoJ</i> for overexpression, introduces XhoI site	GGTCTTTAG ACACTCGAGTTAGTCATCTATCTCCTGTTTGATAATG
up_ykoJ-fw	Forward primer to amplify upstream region of <i>ykoJ</i> , internal pstI site is amplified	CTTTTGCTGCAGCAGCCATTTTAG
up_ykoJ-rv	Reverse primer to amplify upstream region of <i>ykoJ</i> , introduces HindIII site	TCTTAAGCTTTTGTGAGCCCTCCTTTGTTT
down_ykoJ-fw	Forward primer to amplify downstream region of <i>ykoJ</i> , introduces HindIII site	TCTAAGCTTCAGGAGATAGATGACTAATCAA
down_ykoJ-rv	Reverse primer to amplify downstream region of <i>ykoJ</i> , introduces XbaI site	CGATCTAGAAGCATCCAGCTGCATTA
btox-change_FW	Universal forward primer for mutagenesis of β -toxin, introduces Eco31I site	CGGTCTCATATTCATCTGAAATGACAACCTTAATAAAC TTAAC
RV-btox_DAA	Reverse primer, changes DDK \rightarrow DAA	CGGTCTCAAATAAGCAGCATCGATAAATCTAGCATCT ATAGATGCAGTAA
RV-btox_ADA	Reverse primer, changes DDK \rightarrow ADA	CGGTCTCAAATAAGCATCAGCGATAAATCTAGCATCT ATAGATGCAGTAA
RV-btox_AAK	Reverse primer, changes DDK \rightarrow AAK	CGGTCTCAAATATTTAGCAGCGATAAATCTAGCATCTA TAGATGCAGTAA
RV-btox_DDA	Reverse primer, changes DDK \rightarrow DDA	CGGTCTCAAATAAGCATCATCGATAAATCTAGCATCTA TAGATGCAGTAA
RV-btox_DAK	Reverse primer, changes DDK \rightarrow DAK	CGGTCTCAAATATTTAGCATCGATAAATCTAGCATCTA TAGATGCAGTAA
RV-btox_ADK	Reverse primer, changes DDK \rightarrow ADK	CGGTCTCAAATATTTATCAGCGATAAATCTAGCATCTA TAGATGCAGTAA

^a MCS, multiple cloning site.

induced when the culture reached an OD₆₀₀ of ~ 0.5 by the addition of 0.75% of subtilin containing supernatant of strain ATCC 6633 prepared as described previously (4). Two hours after induction, cells were separated from the supernatant by centrifugation for 1 min at 14,000 rpm. Supernatant proteins were concentrated 20-fold following trichloroacetic acid precipitation and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (17). Cell fractions were prepared for SDS-PAGE as described previously (32). Proteins were separated by SDS-PAGE and either stained with Coomassie brilliant blue directly or transferred to a polyvinylidene difluoride membrane (Molecular Probes, Inc., Eugene, OR). β -Toxoid protein was visualized using a monoclonal anti- β -toxoid antibody (Intervet Int., Boxmeer, The Netherlands) and a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Amersham Biosciences, Buckinghamshire, United Kingdom). Protein sizes and concentrations were determined with a prestained protein marker (Fermentas, Vilnius, Lithuania), and Quantity One software (Bio-Rad, Hercules, CA).

DNA microarray experiments. DNA microarray procedures were performed as described by Lulko et al. (20a). In short, RNA was isolated from three independently grown cultures of *B. subtilis* NZ8900 containing either pNRS- β -toxin or pNRS- β -toxoid. β -Toxin or β -toxoid expression was induced as described above, and samples for RNA isolation were taken 1.5 h after induction with subtilin. Single-strand reverse transcription (amplification) and indirect labeling of total isolated RNA with either Cy3 or Cy5 dye were performed, and labeled cDNA samples were hybridized overnight (O/N) at 48°C on in-house-printed microarray slides containing 70-meric oligonucleotides covering all *B. subtilis* open reading frames. After hybridization, slides were washed and scanned. Slide data were processed and normalized as described previously (8), yielding average ratios of gene expression levels of the strain expressing the β -toxoid compared to those of the strain expressing the WT β -toxin. Expression of a gene was considered to be significantly altered when its expression ratio was >1.75 or <0.57 and had a CyberT Bayesian *P* value of <0.001 . All DNA microarray data, including the slide images and raw data, obtained in this study are available online (http://molgen.biol.rug.nl/publication/btox_data/).

PhtrA-GFP and PykoJ-GFP analysis. The *htrA* and *ykoJ* promoter regions were amplified by PCR using primers PhtrA-fw-kpnI and PhtrA-rv and PykoJ-fw and PykoJ-rv, respectively. The PCR products were digested with HindIII and KpnI and ligated into the likewise digested plasmid pDG151. The plasmids were transferred to *E. coli*, and correct clones were checked by PCR and DNA sequencing. The pPhtrA-GFP plasmid was integrated via single crossover in the chromosomal DNA of *B. subtilis* strain NZ8900 at the locus of the *htrA* promoter, creating *B. subtilis* strain HT100A, and the pPykoJ-GFP plasmid was likewise integrated at the locus of the *ykoJ* promoter, creating the *B. subtilis* YkoJ-GFP strain. Green fluorescent protein (GFP) production was measured using a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Mijdrecht, The Netherlands). The average fluorescence of 20,000 gated cells was determined using WinMDI 2.8 (<http://facs.scripps.edu/software.html>) software.

Assay of β -toxin and β -toxoid stability. An O/N culture of *L. lactis* NZ9000 containing either pNZ β tox or pNZ β toxoid was diluted to an OD₆₀₀ of 0.1 and grown for 2.5 h until an OD₆₀₀ of 0.5 was achieved. Nisaplin (stock 50 mg/ml) in a final dilution of 1×10^{-7} was added to induce the nisin-inducible promoter, and 2 h after induction, total supernatant was harvested by centrifugation and subsequent filtration over a 0.2- μ m syringe filter (Schleicher and Schuell Microscience, Dassel, Germany).

To collect spent supernatants of *B. subtilis* strains 168 and WB800, the strains were grown in TY medium, and supernatant samples were taken 2 h into the stationary growth phase. Supernatant was separated by centrifugation and subsequently passed through a 0.2- μ m filter. The β -toxin and β -toxoid samples harvested from *L. lactis* were mixed 1:1 with the spent *B. subtilis* supernatant and incubated for 10 min and 1 h, respectively, at 37°C. As a control, fresh TY medium was used. After incubation, total protein was concentrated 10-fold upon trichloroacetic acid precipitation as described before and analyzed using SDS-PAGE. The concentrations of β -toxin and β -toxoid were determined by Coomassie brilliant blue staining followed by densitometric scanning (Bio-Rad GS-800 scanner) and analysis with Quantity One software (Bio-Rad, Hercules, CA).

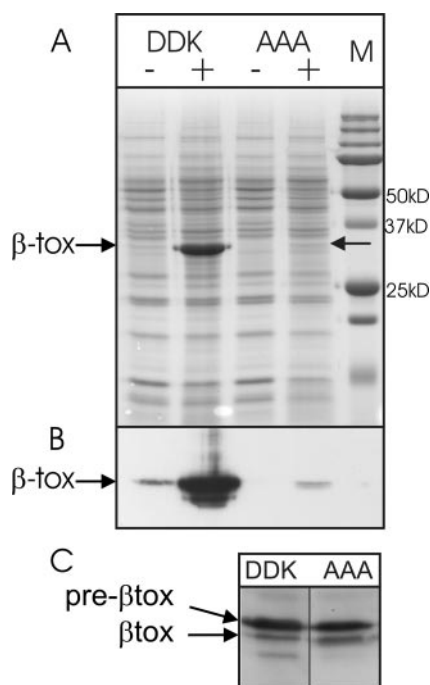


FIG. 1. Production and secretion of β -toxin and β -toxoid. (A) Coomassie brilliant blue-stained 12% SDS-polyacrylamide gel containing 10 \times concentrated supernatant of *B. subtilis* strain NZ8900 1.5 h after induction of the inducible β -toxin/ β -toxoid plasmids. (B) Detection of secreted β -toxin and β -toxoid in the samples shown in panel A, examined by Western blotting using monoclonal antibodies against β -toxin. (C) Detection of intracellular β -toxin and β -toxoid examined by Western blotting using monoclonal antibodies against β -toxin. Pre- β tox and β tox are indicated by the arrows. DDK, WT β -toxin (D₅₄DK); AAA, β -toxoid (A₅₄AA); M, protein marker; +, induction with subtilin; –, no subtilin added.

RESULTS

Recently, we have described the difficulties in secretion of *Clostridium perfringens* β -toxoid by *Bacillus subtilis* (21). Yet, it has been reported that wild-type β -toxin protein can be efficiently expressed in *B. subtilis* (26). To directly compare the secretion efficiencies of both proteins, we cloned them in the subtilin-inducible SURE vector pNZ8903. After induction for 1.5 h, cells were separated from the growth medium, and β -toxin and β -toxoid levels of production and secretion were assayed by SDS-PAGE and Western blotting using monoclonal anti- β -toxin antibody (Fig. 1). Clearly, the β -toxin protein was secreted in high amounts, up to 50% of total extracellular protein, whereas the β -toxoid variant never yielded more than 4%. The intracellular levels of β -toxin and β -toxoid were similar, and both induced cultures showed no significantly decreased growth rate compared to that of the uninduced cultures. Furthermore, no significant growth differences between the β -toxin and β -toxoid production strains were observed (data not shown).

Global gene expression differences between β -toxin and β -toxoid overproduction. As shown in Fig. 1, it is apparent that there is a large difference in the secretion yields of β -toxin and detoxified β -toxoid. To identify possible bottlenecks in the secretion of β -toxoid, we compared the two production strains using DNA microarrays. Either β -toxin or β -toxoid expression was induced, and samples for RNA isolation were taken 1.5 h after induction. Following statistical analysis of the obtained data, several genes showed a clear difference in expression levels between the two strains. An overview of the results of the DNA microarray is presented in Table 3.

TABLE 3. DNA microarray results

Gene	Ratio ^c	Bayesian <i>P</i> value	Function (putative) ^d
Upregulated genes^a			
<i>ykoJ</i>	6.87	2.25E-14	Unknown
<i>yvtA</i>	2.66	1.47E-10	<i>htrB</i> , serine protease, secretion stress
<i>htrA</i>	2.39	6.06E-10	Serine protease, secretion stress
<i>spollR</i>	2.33	0.000902	Sporulation
<i>ykzD</i>	2.28	5.95E-05	Unknown, directly downstream of <i>ykoJ</i>
<i>yvqH</i>	2.09	3.12E-06	<i>lial</i> , part of <i>liaRS</i> regulon
<i>cggR</i>	1.97	0.000437	Transcriptional repressor of <i>gapA</i>
<i>yvqI</i>	1.95	2.15E-05	<i>liaH</i> , part of <i>liaRS</i> regulon
<i>ldh</i>	1.92	0.000111	L-Lactate dehydrogenase
<i>mtlD</i>	1.92	6.48E-06	PTS, mannitol-specific enzyme
<i>mtlA</i>	1.88	2.57E-06	PTS, mannitol-specific enzyme
<i>cssR</i>	1.76	0.000847	Two-component regulator, secretion stress
Downregulated genes^b			
<i>purN</i>	-2.61	6.84E-11	Purine biosynthesis
<i>purM</i>	-2.55	1.06E-05	Purine biosynthesis
<i>purH</i>	-2.47	1.49E-08	Purine biosynthesis
<i>rocR</i>	-2.12	1.89E-08	Regulator, binding box upstream of <i>ykoJ</i>
<i>yomM</i>	-1.94	0.00081	Unknown
<i>yddE</i>	-1.9	3.65E-05	Unknown, operon on transposon region
<i>yddB</i>	-1.86	3.72E-05	Unknown, operon on transposon region
<i>yecA</i>	-1.85	0.000455	Transport/binding proteins and lipoproteins
<i>ydcP</i>	-1.8	0.000163	Unknown, operon on transposon region
<i>yosP</i>	-1.79	0.000742	Unknown, operon on transposon region
<i>yddF</i>	-1.77	0.0003	Unknown, operon on transposon region

^a Upregulated genes, higher expression when β -toxoid expression is compared to β -toxin expression.

^b Downregulated genes, lower expression when β -toxoid expression is compared to β -toxin expression.

^c Ratio, expression ratio comparing expression level for strain NZ8900 overproducing β -toxoid (target strain) to that for NZ8900 overexpressing β -toxin (control).

^d PTS, phosphotransferase system.

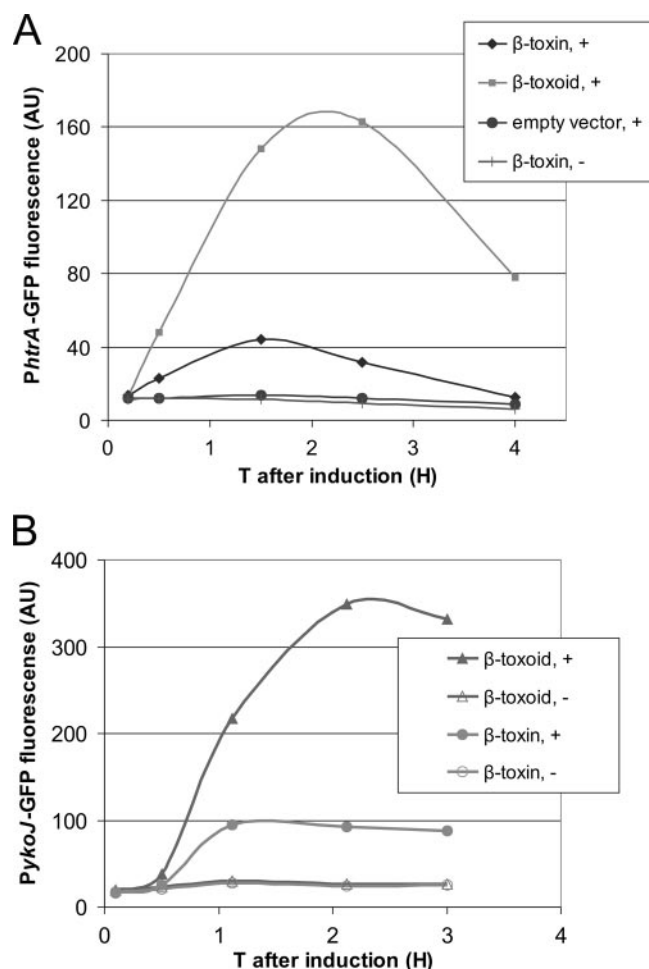


FIG. 2. (A) *PhtrA*-GFP upon overexpression of β -toxin and β -toxoid, shown by internal GFP fluorescence over time (T) of *B. subtilis* HT100A containing either β -toxin or β -toxoid on an inducible plasmid. After induction at T_0 of β -toxin/ β -toxoid, the response of the *htrA* promoter was measured by quantifying the average GFP fluorescence per cell using a flow cytometer. (B) *PykoJ*-GFP upon overexpression of β -toxin and β -toxoid, shown by internal GFP fluorescence over time of the *B. subtilis* *PykoJ*-GFP strain in its chromosome and either β -toxin or β -toxoid on an inducible plasmid. After induction at T_0 of β -toxin/ β -toxoid, the response of the *ykoJ* promoter was measured by quantifying the average GFP fluorescence per cell using a flow cytometer. Cultures not induced were also measured. +, induced; -, not induced.

The most strongly upregulated gene in the β -toxoid-expressing strain was *ykoJ*, a gene of unknown function. Furthermore, *htrA* and *htrB* were upregulated, as was *cssR*, part of the two-component system known to regulate *htrA* and *htrB* expression (7). An upregulation of *htrA* upon expression of β -toxoid, as monitored by a *PhtrA-lacZ* fusion, was previously described (21). Clearly, this upregulation is lower for the wild-type β -toxin, since a difference in *htrA* expression is found in the array comparison. An overview of the other differentially expressed genes and their (putative) functions is given in Table 3.

To validate the DNA microarrays, we looked specifically at the expression of *ykoJ* and *htrA* by using promoter GFP reporter fusions. We measured the average GFP expression per cell using flow cytometry. As shown in Fig. 2A, *htrA* expression

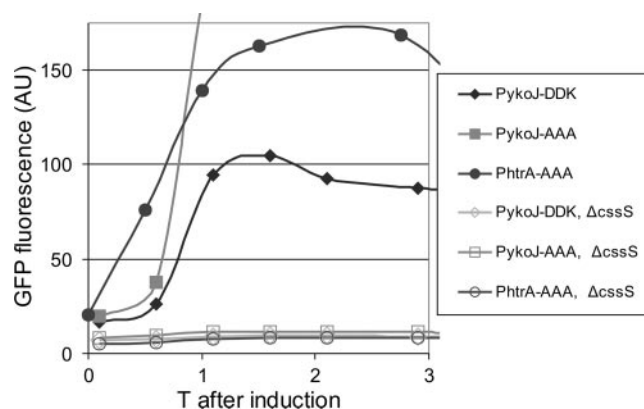


FIG. 3. Effect of *cssS* disruption on responses of *PykoJ*-GFP and *PhtrA*-GFP strains. Internal fluorescence of *B. subtilis* HT100A or the *PykoJ*-GFP strain was measured. Response was determined with or without a *cssS* disruption in the strain. In all strains, β -toxin (D_{54} DK) or β -toxoid (A_{54} AA) was induced by the addition of subtilin at T_0 .

in a strain that expressed β -toxoid was three times higher than that in a strain that expressed β -toxin. It should be noted that without induction, the *PhtrA*-GFP levels are much lower, indicating that expression and secretion of β -toxin also causes secretion stress. As shown in Fig. 2B, the results obtained with a *PykoJ*-GFP fusion confirmed the transcriptome result as well. Like *htrA*, *PykoJ* was moderately upregulated when β -toxin was induced and strongly upregulated when β -toxoid was induced.

Since the expression of *ykoJ* resembled the expression pattern of *htrA*, we tested whether the expression of *ykoJ* was controlled by the CssRS two-component system. For this test, a *cssS* disruption (13) was introduced into the *PykoJ*-GFP reporter strain. As shown in Fig. 3, no response of the *ykoJ* promoter occurred upon induction of β -toxin or β -toxoid when *cssS* was mutated. Together, the microarray analysis data show that the induction of the secretion stress regulon governed by CssRS is most apparent upon induction of β -toxoid.

YkoJ deletion does not improve β -toxoid secretion. The transcriptome data suggest that the CssRS regulon could be a target when improving secretion of β -toxoid. In a previous study we already showed that the mutation of the CssRS two-component system does not positively effect production of β -toxoid (21). Since a mutation of CssRS will effectively prevent the induction of HtrA and HtrB (7), deletion of these two induced proteases separately is unlikely to improve secretion of β -toxoid. In studies performed by Vitikainen et al. (33), it was also observed that downregulation or mutation of HtrA and/or HtrB proteases does not improve secretion but instead induces severe stress in the cells, resulting in poor growth and generally lower secretion yields. However, Since *ykoJ* was strongly overexpressed in our study, we tested whether this protein itself influences the efficiency of secretion of β -toxoid. YkoJ contains two PepSY domains that suggest a peptidase-inhibiting action (36), but the specific function of YkoJ is still unknown. A deletion of *ykoJ* showed no noteworthy improvement of β -toxoid secretion. Also, the secretion level of β -toxin in this strain did not differ from that of the wild-type strain (data not shown). We constructed a YkoJ overproduction

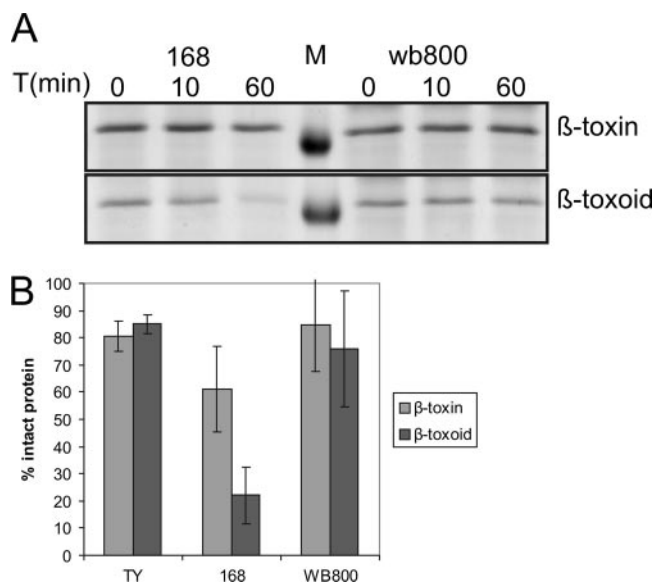


FIG. 4. Degradation of β -toxin and β -toxoid by spent *B. subtilis* supernatants. *Lactococcus lactis*-produced β -toxin and β -toxoid cultures were incubated with spent supernatants of stationary-phase cultures of *B. subtilis* strains 168 and WB800. As a control, TY medium was used. (A) Typical Coomassie brilliant blue-stained polyacrylamide gel showing results of the degradation assay. The incubation time (T) in minutes is indicated. Left lanes show β -toxoid and β -toxin exposed to strain 168 culture supernatant; right lanes show β -toxoid and β -toxin exposed to strain WB800 culture supernatant. M, protein marker, 35-kDa band. (B) The amount of β -toxin/ β -toxoid measured after 10 min was set to 100%. The remaining amounts of β -toxoid and β -toxin after 1 h were determined and plotted. Experiments were performed in duplicate; error bars depict standard errors.

strain using a SURE expression system. Unfortunately, upon mild induction, the cultures stopped growing and started lysing, indicating that the overexpression of *ykoJ* is lethal to *B. subtilis* (data not shown). These results indicate that *YkoJ* alone is not directly involved in the large difference in secretion level between β -toxin and β -toxoid.

Amino acid differences between β -toxin and β -toxoid. Since altering the expression of host genes did not improve the yield of secreted β -toxoid, we focused on the nature of the protein itself. The differences between β -toxin and β -toxoid are three consecutive mutations at the N-terminal side of the mature protein (D54A, D55A, and K56A). Based on a homology model of the mature β -toxin protein available at the MODBASE protein model database (22), we have looked at the positions of these residues in the folded protein. According to the model, β -toxin consists largely of β sheets. However, the residues 54, 55, and 56 (as counted from the first residue of the mature protein) are situated in a loop at the surface of the protein and consist of two negatively charged aspartic acids and a positively charged lysine. In β -toxoid, these residues are replaced with alanines. It is likely that this change in charge distribution affects the folding characteristics of the protein.

Since the amino acid substitutions in the β -toxin mutants might influence folding and stability of the protein, we tested its susceptibility to proteases. β -Toxoid and β -toxin produced by *L. lactis* were incubated with spent supernatant of a station-

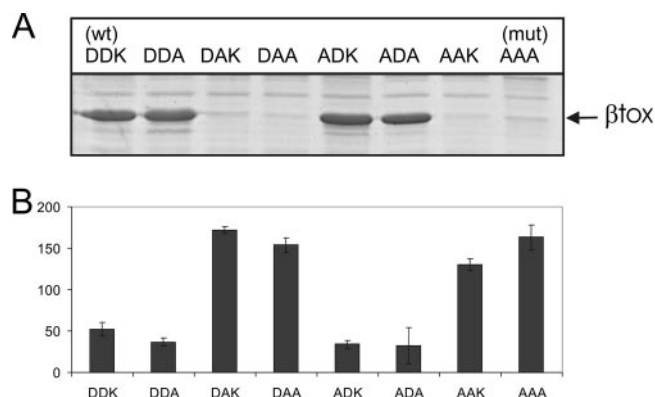


FIG. 5. Secretion and secretion stress of β -toxin, β -toxoid, and intermediate mutants. (A) Coomassie brilliant blue-stained 12% SDS-polyacrylamide gel containing 10 \times concentrated supernatant of *B. subtilis* strain NZ8900 1.5 h after induction of the inducible β tox plasmids. (B) The average *PhrA*-GFP expression per cell in arbitrary units, measured 1.5 h after induction, is shown. Experiments were performed in duplicate; error bars depict standard errors. DDK, WT β -toxin (D₅₄DK); AAA, β -toxoid (A₅₄AA); all other intermediate mutants (mut) are likewise indicated.

ary-phase *B. subtilis* culture. This culture supernatant contains many proteases secreted by *B. subtilis*. As shown in Fig. 4, a clear difference between the stabilities of the two proteins is visible. Whereas more than 50% of the β -toxin is still present after 1 h of incubation, almost all β -toxoid (>90%) has been degraded. As a control, we tested supernatant from *B. subtilis* WB800. In this strain, the genes for eight proteases have been deleted (35). Incubation with supernatant from a WB800 culture gave significantly less degradation, and about 60% β -toxoid was still detectable after 1 h of incubation (Fig. 4). The results show that β -toxoid is more prone to degradation than β -toxin, indicating that the amino acid substitutions do make the protein conformation less stable.

Intermediate mutants between β -toxin and β -toxoid. To examine the importance of the individual amino acid mutations for protein secretion, we constructed all six possible intermediate mutants. Expression and secretion of the different β -toxin variants was tested by harvesting cells and supernatant fractions 1.5 h after induction and analyzing them by SDS-PAGE and Western blotting (Fig. 5A). From these experiments, two classes emerged: those with β -toxin production levels (D₅₄DK [β -toxin], D₅₄DA, A₅₄DA, and A₅₄DK) and those with hardly any protein secreted (A₅₄AA [β -toxoid], A₅₄AK, D₅₄AK, and D₅₄AA). This screening indicated that mutations at residues 54 and 56 do not have a significant effect on secretion efficiency, yet residue D₅₅ is pivotal when it comes to efficient secretion of toxin.

The mean lethal doses of the β -toxin (D₅₄DK) and the β -toxoids (D₅₄DA, A₅₄DA, and A₅₄DK) were assessed by intravenous injection, in sterile phosphate-buffered saline, into mice weighing approximately 25 g. All three β -toxoids had a similar level of toxicity which is approximately one-fifth of that of the toxin. The original β -toxoid (A₅₄AA) has a toxicity that is approximately 30-fold lower than that of the wild-type toxin (24). The poorly secreted β -toxoids (A₅₄AK, D₅₄AK, and D₅₄AA) were not tested, since production levels were too low.

We also measured the effect of these six intermediate mutants on *htrA* expression. In accordance with the previous results, all mutants with an aspartic acid at position 55 showed relatively low, β -toxin-like *htrA* expression levels, whereas the mutants with an alanine at this position showed a strong up-regulation of *htrA*, comparable to that for β -toxoid production (Fig. 5B). Clearly there is a strong relationship between poor β -toxoid secretion and the induction of the secretion stress regulon.

DISCUSSION

β -Toxoid, the genetically altered variant of *Clostridium perfringens* β -toxin, is not efficiently produced by *Bacillus subtilis*. To improve the secretion yield of the β -toxoid protein, we swapped signal sequences, used several expression systems, and tested protease-deficient hosts (21). None of the tested methods resulted in an appreciable increase in yield. However, wild-type β -toxin could be secreted much better than β -toxoid, in yields exceeding 50% of the total secreted protein fraction. To identify the bottleneck that was causing this difference in secretion yields, we applied a genome-wide expression analyses of the two production strains, hoping to find genes or processes responsible for this large production difference.

The DNA microarray analysis revealed that the differences can be largely attributed to the C_{ss}RS regulon, an indication of unfolded protein stress. The most upregulated gene in our array study, *ykoJ*, appeared to be part of the C_{ss}RS regulon as well. A deletion of the C_{ss}S sensor, effectively preventing induction of *htrA*, *htrB* (7), and *ykoJ*, did not improve secretion. We tried to overproduce YkoJ, but this proved to be lethal. Next to the C_{ss}RS regulon, two genes present in the *lia*RS regulon (15) were expressed significantly higher in the β -toxoid mutant. This effect was also found in another secretion stress study (2). Recently, it has been shown that LiaRS is activated by cell envelope stress (15). Only *liaI* and *liaH*, the genes that are generally much more highly expressed than the other genes in the regulon (15), were significantly upregulated in our study. The other genes that are part of this regulon were not found, indicating that the LiaRS induction differences are minor in our transcriptome comparison. We therefore did not characterize the effect of *lia*RS on β -toxoid production. Several of the purine biosynthesis genes were found to be downregulated, indicating a slight decrease in growth rate, which was missed in the growth rate determination but is picked up by the more sensitive microarray analysis.

Since altering the production host to increase secretion of β -toxoid was so far not successful, we looked more closely at β -toxoid itself, as the differences in yield between β -toxin and β -toxoid were striking. The stretch of three amino acid substitutions that morphs β -toxin into β -toxoid is not located in the secretion signal peptide where point mutations can have large effects on secretion efficiency (37). Furthermore, levels of intracellular retention of both the β -toxin and the β -toxoid are similar, indicating that no stalling problems occur when the protein gets secreted over the cytoplasm membrane via the Sec translocon.

Proteins secreted via the Sec secretion pathway are generally thought to be secreted in an unfolded state and are folded only after secretion over the plasma membrane (31). The current

model of the β -toxin protein suggests that the point mutations introduced in β -toxoid might interfere with the correct folding or the rate of folding of β -toxoid after secretion. Upon induction of β -toxoid, a secretion stress response is observed, most likely induced by unfolded, secreted protein (5, 13). These results suggest that β -toxoid is reaching the outside of the membrane. The exact signal sensed by the C_{ss}S secretion stress sensor is as yet unknown, as it could also be the breakdown products of the misfolded and degraded protein that trigger the system.

The changed residues in β -toxoid are most likely affecting optimal folding kinetics, and therefore the β -toxoid protein is much more prone to degradation. Our experiments validated this assumption and showed that β -toxoid was much more prone to proteolysis than β -toxin, indicating that β -toxoid is in a folded conformation that is less stable than the WT β -toxin. The tested β -toxoid was produced and secreted by *L. lactis*, which could have influenced the folding of this protein. However, this is likely to be equally true for the β -toxin, which also was produced by *L. lactis* and which justifies this comparison. Incubation with the supernatant of *B. subtilis* strain WB800, which lacks the genes for seven extracellular proteases and the cell wall protease WprA, resulted in considerably less breakdown of β -toxoid. However, expression of β -toxoid by *B. subtilis* WB800 resulted in only a minimal improvement of β -toxoid secretion (21). This demonstrates that in the case of β -toxoid, most of the secreted protein is degraded before it is targeted by WprA or the other extracellular proteases deleted in WB800.

The constructed intermediate mutants of β -toxin demonstrate that only the aspartic acid at position 55 is necessary for the high secretion of the β -toxin. Although residues 54 and 56 also are charged and locate at the outside of the protein, they seem to be unimportant for secretion efficiency. They do play a role in the toxicity of the β -toxin, since the alterations of these residues does lower toxicity fivefold. The reason for this we do not know; possibly future structural studies might clarify this.

The responses of the *htrA* and *ykoJ* promoters to the overproduction of β -toxoid is indicative of extracellular folding stress. As proposed by Westers et al. (34), the expression of *PhtrA* or *PhtrB* can be utilized to monitor protein secretion. This study has added the *ykoJ* promoter to the possible indicators of secretion stress. A screening method using this promoter and site-directed/random mutagenesis of the secreted substrate should provide a rapid method to improve heterologous protein secretion.

With this study, we present a case where the bacterial host can be adapted in many ways without a significant yield improvement of secreted heterologous protein. The bottleneck turned out to be the secreted protein itself, where one point mutation made a crucial difference. In many cases, the intrinsic properties of the heterologous protein can be a main cause of the limited production yields, and increased attention to optimizing the protein itself rather than only the expression host is required.

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